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Impact of interleukin-1 receptor antagonist and tumor necrosis factor-α gene polymorphism on IgA nephropathy

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Background. It is evident that cytokines play an important role in the pathogenesis as well as disease progression in IgA nephropathy (IgAN). The level of cytokine production is influenced by different genotypes that reflect gene polymorphism of the pertinent cytokine. Interleukin-1 receptor antagonist (IL-1ra) and tumor necrosis factor-α (TNF-α) gene polymorphism have been found to affect disease susceptibility and activity in several inflammatory diseases. However, the impact of these polymorphisms in IgAN patients has not previously been thoroughly studied.

Methods. We investigated 111 cases of biopsy-proven IgAN and 100 healthy, normal controls for their IL-1ra and TNF-α gene polymorphism. IL-1ra gene polymorphism was characterized as a variable number of tandem repeats of a 86 bp sequence within intron 2. Five alleles were identified and were designated as IL1RN*1, IL1RN*2, IL1RN*3, IL1RN*4, and IL1RN*5, corresponding to 4, 2, 5, 3, 6 repeats, respectively. A polymorphism in the promoter region of the TNF-α gene was also studied. This polymorphism involved a guanidine to adenosine transition at position −308 and was designated as TNF1 (−308G) and TNF2 (−308A).

Results. There were 54 male and 57 female patients with a mean age of 30.3 ± 12.5 years and a disease duration of 66.8 ± 47.2 months. The mean duration of the follow-up period was 47.3 ± 32.6 months. In the patient group, the allele frequencies of IL1RN*1, IL1RN*2, IL1RN*3, IL1RN*4, and IL1RN*5 were 89.6%, 9.9%, 0%, 0.5%, and 0%, respectively, whereas the corresponding carriage rates were 100%, 19.8%, 0%, 0.9%, and 0%, respectively. An excessive carriage of IL2RN*2 was found in the patients when compared with normal controls (allele frequency, 9.9 vs. 2.5%, $P < 0.0001$). The allele frequencies of TNF1 and TNF2 were 94.1 and 5.9%, respectively, and the carriage rates were 99.1 and 10.8%, respectively, in the patients, which was not significantly different from those of normal controls. When the patients were stratified into mild and severe groups according to their initial presentation, none of the studied alleles correlated with the severity. However, patients with gross hematuria were associated with a higher carriage rate of TNF2 when compared with patients without gross hematuria (allele frequency, 15.4 vs. 4.6%, $P = 0.0552$; carriage rate, 30.8% vs. 8.2%, $P = 0.0272$). Renal survival analysis revealed that the TNF2 carrier had a renal survival comparable with TNF2 (−) patients. However, the carriage of the IL1RN*2 allele was associated with a significantly poorer long-term outcome with a median survival time of 72 months, as compared with those without IL1RN*2 (134 months, $P < 0.01$).

Conclusion. IL-1ra and TNF-α gene polymorphism may affect disease susceptibility as well as disease activity and long-term outcome in human IgAN. Treatment with an IL-1ra or IL-1 blocking agent may be relevant in those carrying the IL1RN*2 allele.

Key words: cytokine gene polymorphism, progressive renal disease, glomerulonephritis, inflammation.

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IgA nephropathy (IgAN) is the most common primary glomerulonephritis in many countries, including Taiwan. The pathogenetic mechanism is still speculative and controversial [1]. Nevertheless, a great body of evidence suggests that cytokines play an important role in the pathogenesis and disease progression of IgAN [2–6]. Interleukin-1 (IL-1) has been found to be expressed in the glomeruli of IgAN patients [4, 6], and this may be a marker of glomerular mesangial cell activation [5] in response to a nephritogenic immune complex. It had been shown that IL-1 is involved in mesangial cell proliferation [2] and extracellular matrix production [7]. IL-1 receptor antagonist (IL-1ra), a glycoprotein generated by monocytes/macrophages and polymorphonuclear cells, can inhibit IL-1 activity by competing for receptor binding [8, 9]. IL-1ra has been shown to suppress experimental glomerulonephritis [10, 11] and IgAN [12]. The *IL-1ra* gene has been found to have a variable number of tandem repeat polymorphism in intron 2 [13]. The presence of the two-repeat allele (IL1RN*2) in individuals has been shown to be associated with several inflammatory diseases, such as psoriasis [14], alopecia areata [15], ulcerative colitis [16], systemic lupus erythematosus [17], Graves' disease [18], as well as diabetic complication [19]. Recently, an excessive carriage of IL1RN*2 was found in IgAN patients with gross hematuria and Henoch-Schönlein purpura [20], suggesting a genetic link

between these two diseases. The impact of carriage of IL1RN*2 on the long-term outcome in IgAN, however, has yet to be addressed.

Tumor necrosis factor-α (TNF-α) is also a proinflammatory cytokine that has been involved in the pathogenesis of glomerulonephritis, including IgAN [4, 6, 21]. A gene polymorphism at position −308 in the promoter region involving a transition of guanidine to adenosine has been characterized [22]. The −308A polymorphism (TNF2) is associated with an increased of TNF-α transcription [23], which may predispose humans to the occurrence of inflammatory diseases. Carriage of TNF2 has been shown to be associated with several inflammatory diseases, including alopecia areata [24], rheumatoid arthritis [25], and systemic lupus erythematosus [26]. The effect of TNF2 on the disease activity in IgAN has not been studied before. The aim of the present study was to define the impact of IL-1ra and TNF-α gene polymorphism on disease activity as well as on the long-term outcome of IgAN.

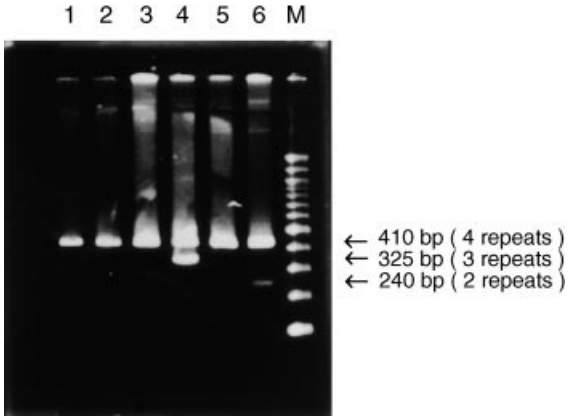

Fig. 1. Electrophoresis of the polymerase chain reaction (PCR) product of the interleukin-1 receptor antagonist (*IL-1ra*) gene. Lanes 1, 2, 3, and 5 are four-repeat (410 bp) homozygotes. Lane 4 is a heterozygote of four repeats and three repeats (325 bp). Lane 6 is a heterozygote of four repeats and two repeats (240 bp). M = DNA marker ladder.

METHODS

Patients

Patients with biopsy-proven IgAN were retrospectively studied for their IL-1ra and TNF-α gene polymorphism. To enroll only those with a longer term of follow-up, patients undergoing biopsy after 1995 were excluded. Up to 1994, there were 215 cases of biopsy-proven IgAN, of which only 111 cases had a clear-cut history and adequate follow-up data to be analyzed and formed the basis of this study. Patients were either referred from local clinics or were seen in our hospital from the very beginning. It is our policy that all of the patients with unexplained hematuria and/or proteinuria were advised to have a kidney biopsy. Informed consent was routinely obtained for every patient undergoing kidney biopsy. According to the initial clinical presentation, patients were stratified into two subgroups: mild, meaning a serum creatinine level of less than 1.3 mg/dL, a daily urine protein loss of less than 1.5 g, and normal blood pressure, and severe, meaning the presence of a serum creatinine level >1.3 mg/dL, a daily urine protein loss >1.5 g, or hypertension. To evaluate the impact of gene polymorphism on disease progression, patients were subdivided into progressor and nonprogressor groups according to their renal function profile at the last follow-up examination. Progressor meant patients with any of the following pictures during follow-up: a steady increase of serum creatinine level, a 50% or more increase of daily urine protein loss as compared with the baseline value, or the appearance of hypertension in a previously normotensive patient. Nonprogressor meant patients who had a stable renal disease or those who were in remission. Disease duration was defined as the duration between the apparent onset of disease, such as gross hematuria or abnormal urinalysis, and the end of the follow-up period. The duration of follow-up was defined as the duration between kidney biopsy and the end of the follow-up period. Because a few cases were presented as end-stage renal failure without a clear-cut history, the previously mentioned duration was given as 0.

Polymerase chain reaction

Genomic DNA was extracted from peripheral blood mononuclear cells using DNAzol reagent (GIBCO BRL, Grand Island, NY, USA). IL-1ra genotyping was based on the method by Tarlow et al [13] and TNF-α genotyping according to Wilson et al [22]. Briefly, the polymerase chain reaction (PCR) consisted of a denaturing at 94°C for two minutes, then 30 cycles at 94°C for 30 seconds, 60°C for 30 seconds, and 72°C for 45 seconds, and a final extension at 72°C for five minutes. The following primers were used: IL-1ra, 5′-CTCAGCAACACTCCTAT-3′ and 5′-CCTGGTCTGCAGGTAA-3′; TNF-α, 5′-AGGCAA TAGGTTTTGAGGGCCAT-3′ and 5′-TCCTCCCTGC TCCGATTCCG-3′. The PCR products for IL-1ra were analyzed by electrophoresis on 2% agarose gels and were stained with ethidium bromide for visualization under ultraviolet light. The polymorphism was based on the number of repeats of an 86 bp sequence [13] and were designated as IL1RN*1 (four repeats, 410 bp), IL1RN*2 (two repeats, 240 bp), IL1RN*3 (five repeats, 500 bp), IL1RN*4 (three repeats, 325 bp), and IL1RN*5 (six repeats, 595 bp) (Fig. 1). PCR products for TNF-α were digested overnight with NcoI (NEB) at 37°C and were analyzed on 4% NuSieve 3:1 agarose gels (FMC Bioproducts, Rockland, ME, USA). The polymorphism was designated as TNF1 (−308G), which gave two fragments

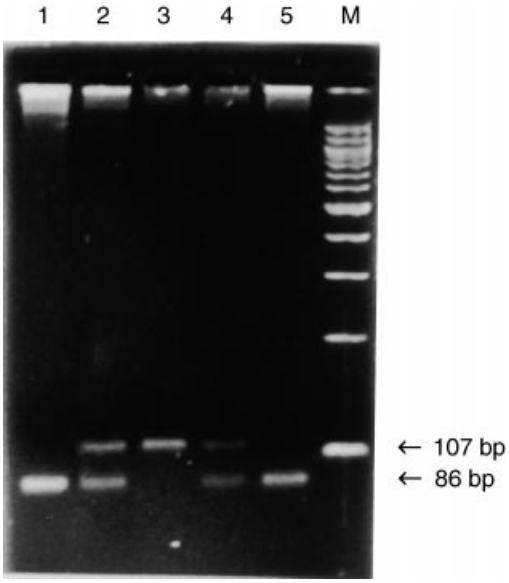

Fig. 2. Electrophoresis of the PCR product of the tumor necrosis factor-α (*TNF-α*) gene. Lanes 1 and 5 are homozygotes of TNF1 (−308G). Lane 3 is a homozygote of TNF2 (308A), and lanes 2 and 4 are heterozygotes of TNF1 and TNF2.

of 87 bp and 20 bp, and TNF2 (308A), which gave a single 107 bp fragment (Fig. 2).

Statistics

Allele frequency was calculated as the number of occurrences of the test allele in the population divided by the total number of alleles. The carriage rate was calculated as the number of individuals carrying at least one copy of the test allele divided by the total number of individuals. The correlation between polymorphism and clinical parameters was analyzed using Fisher's exact test. A Bonferoni correction was made for multiple comparisons. Renal survival analysis in patients carrying the specific allele was performed by Kaplan–Meier's method and was compared with a log rank test.

RESULTS

Table 1 gives the patients' characteristics. A total of 111 cases, including 57 male and 54 female patients, was enrolled. The mean duration of disease was 66.8 ± 47.2 months (range, 0 to 288 months), and the duration of follow-up was 47.3 ± 32.6 months (range, 0 to 132 months). During the follow-up period, 45 out of 111 cases (40.5%) were classified as progressors. Of these, 34 patients reached end-stage renal failure and were on maintenance dialysis, and 3 patients had predialysis advanced renal failure (serum creatinine > 5 mg/dL); 3 were in modest renal failure (serum creatinine 2 to 5 mg/dL), and 5 had mild renal insufficiency (serum creatinine between 1.3 and 2.0 mg/dL). All of the progressors

Table 1. Patient demographics

	Total
Number	111
Sex *M*:*F*	57:54
Age *years* (range)	30.3 ± 12.5 (10–69)
Disease duration *months* (range)	66.8 ± 47.2 (0–288)
Follow-up duration *months* (range)	47.3 ± 32.6 (0–132)

Table 2. Allele frequency and carriage rate

	Allele frequency %		Carriage rate %	
	Control	IgAN	Control	IgAN
IL1RN*1	96.5	89.6	99.0	100
IL1RN*2	2.5	9.9[a]	4.0	19.8[a]
IL1RN*3	0	0	0	0
IL1RN*4	1.0	0.5	2.0	0.9
IL1RN*5	0	0	0	0
TNF1	88.0	94.1	98.0	99.1
TNF2	12.0	5.9	21.0	10.8

[a] $P < 0.0001$

were accompanied with hypertension and significant degrees of proteinuria (>1.5 g/day). There was no statistically significant difference between progressors and nonprogressors in terms of sex distribution, disease duration, and duration of follow-up. The allele frequency and carriage rate are shown in Table 2. The observed distribution of homozygotes and heterozygotes conformed to Hardy–Weinberg expectations. Most patients carried IL1RN*1 and TNF1 (carriage rate, 100 and 99.1%, respectively). An excessive carriage of IL1RN*2 was found in the patient group as compared with the normal controls (allele frequency, 9.9 vs. 2.5%, $P < 0.0001$; carriage rate, 19.8 vs. 4.0%, $P < 0.0001$). When the patients were stratified into subgroups, there was a trend toward an increased allele frequency and carriage rate of IL1RN*2 among those with gross hematuria and with progressive disease, although this phenomenon did not reach statistical significance. However, patients with gross hematuria were associated with a higher rate of TNF2 carriage as compared with those did not have gross hematuria (allele frequency, 15.4 vs. 4.6%, $P = 0.0552$; carriage rate, 30.8 vs. 8.2%, $P = 0.0272$; Table 3 and Fig. 3). When the renal survival rate was analyzed, the carriage of TNF2 had no impact on the survival (Fig. 4). However, patients who carried IL1RN*2 had a significantly shorter period of long-term survival as compared with those without IL1RN*2 (Fig. 5). The median survival time was 134 months for the IL1RN*2 (+) group and 72 months for the IL2RN*2 (−) group ($P < 0.01$).

DISCUSSION

Interleukin-1 receptor antagonist is an 18 kD glycoprotein produced by macrophages/monocytes and poly-

Table 3. Association of allele frequency with clinical parameters

		Allele frequency %				Carriage rate %			
		IL1RN*2		TNF2		IL1RN*2		TNF2	
	N	+ve	−ve	+ve	−ve	+ve	−ve	+ve	−ve
Mild	87	9.8	90.2	6.3	93.7	19.5	80.5	11.5	88.5
Severe	24	10.4	89.6	4.2	95.8	20.8	79.2	8.3	91.7
With gross hematuria	13	15.4	84.6	15.4[a]	84.6	30.8	69.2	30.8[b]	69.2
Without gross hematuria	98	9.2	90.8	4.6	95.4	18.4	81.6	8.2	91.8
Progressor	45	12.2	87.8	4.4	95.6	24.4	75.6	8.9	91.1
Nonprogressor	66	8.7	91.5	8.9	90.1	16.9	83.1	12.3	87.7

[a]$P = 0.0276$, [b]$P = 0.0136$ as compared to those without gross hematuria

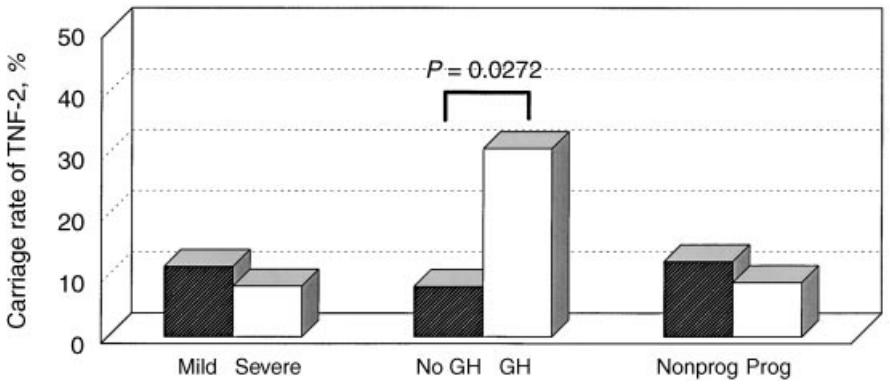

Fig. 3. Carriage rate of TNF2 (−308A) in different subgroups of IgA nephropathy (IgAN) patients. A significantly higher rate was observed in patients with a history of gross hematuria (GH). Abbreviations are: prog, progressors; nonprog, nonprogressors.

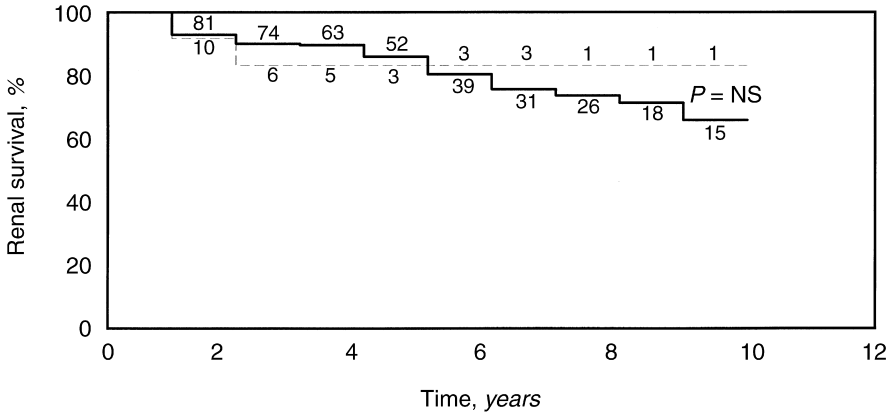

Fig. 4. Renal survival analysis in IgAN patients with or without TNF2. There was no significant difference between TNF2 (+) (dashed line) and TNF2 (−) (solid line) patients. The numbers on the graph represent numbers of patients remaining at each time point.

morphonuclear cells [8, 9], and it is structurally related to IL-1α and IL-1β with a homology in amino acid sequence of 19 and 26%, respectively. It specifically inhibits IL-1 activity by competing for receptor binding and is one of the most powerful endogenous anti-inflammatory agents. Recombinant IL-1ra has been found to suppress experimental crescentic GN [10, 11] and IgAN in mice [12]. Studies of the *IL-1ra* gene have documented a polymorphism within intron 2. Five different alleles have been characterized according to the number of tandem repeats of an 86 bp sequence [13]. Individuals carrying the IL1RN*2 allele were found to be associated with psoriasis [14], alopecia areata [15], and ulcerative colitis [16]. In patients with systemic lupus erythematosus, the IL1RN*2 carrier showed a picture of more intensive disease, in particular with photosensitivity and discoid skin lesions [17]. In diabetes mellitus patients, the carriage of IL1RN*2 was associated with an increased risk of diabetic complications, especially diabetic nephropathy [19]. The mechanism underlying this association is still speculative. It was thought that individuals carrying IL1RN*2 might produce less IL-1ra [19], thereby being predisposed to inflammation. However, the levels of intracellular IL-1ra mRNA from keratinocytes with differ-

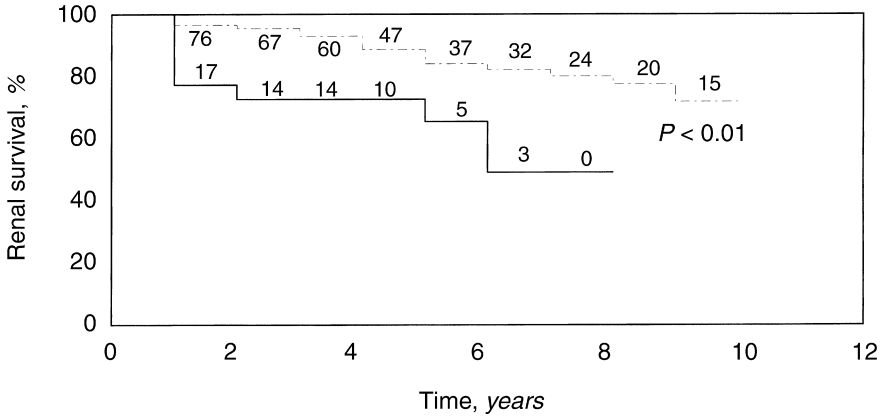

Fig. 5. Renal survival analysis in IgAN patients with (solid line) or without (dashed line) IL1RN*2. Patients carrying IL1RN*2 were associated with a significantly shorter length of long-term survival; the median survival time for IL1RN*2(−) was 134 months and for IL1RN*2(+) was 72 months.

ent intron two alleles were not significantly different [27]. In contrast, increased IL-1ra synthesis had been demonstrated in diabetic patients [28] and normal individuals carrying IL1RN*2 [29, 30]. However, the enhancing effect of IL1RN*2 on plasma IL-1ra level requires the presence of the IL-1β-511 allele 2 or the absence of the IL-1β +3953 allele 2, suggesting that the IL-1β gene participates in the regulation of IL-1ra production [30]. It is speculated that individuals carrying IL-1β-511 allele 2 or with the absence of the IL-1β +3953 allele 2 produce an abundance of IL-1, which, in turn, triggers a strong IL-1ra production in the IL1RN*2 carriers because IL-1 is a strong inducer of IL-1ra [31]. Therefore, IL1RN*2 is probably a marker for a linked disease-associated locus rather than a direct disease-associated allele. Studies with normal control subjects showed that IL1RN*2 was associated mainly with disease severity rather than susceptibility. Recently, an increased rate of IL1RN*2 carriage was found in IgAN patients with gross hematuria and Henoch-Schönlein purpura, leading to the postulation of a genetic link between these two groups of patients [20]. The current study also showed a higher allele frequency of IL1RN*2 (15.4%) among those with gross hematuria as compared with those without gross hematuria (9.2%), although this did not reach statistical significance and was probably caused by a relatively small population of those with gross hematuria ($N = 13$). Interestingly, for the first time, we demonstrated a significantly increased allele frequency and a carriage rate of IL1RN*2 in patients with IgAN and a lower long-term renal survival rate among IgAN patients carrying IL1RN*2.

The role of IL-1 in the pathogenesis of IgAN has been thoroughly studied. IL-1 promotes mesangial cell proliferation [2] and extracellular matrix production [7]. IL-1 can be produced either locally in the glomeruli [32] or by peripheral blood mononuclear cells [33] in patients with IgAN. A phenotypic study of cytokine expression in the kidneys of IgAN patients found that IL-1 was one of the most commonly identified cytokines other than IL-6, TNF-α, interferon-γ, and platelet-derived growth factor [6]. The number of glomerular infiltrating macrophages/monocytes positive for IL-1α correlated with mesangial cell proliferation, whereas interstitial cells positive for IL-1α correlated with the grade of tubulointerstitial changes and proteinuria in IgAN patients [4]. Increased excretion of TNF-α and IL-1β in urine from patients with IgAN and Henoch-Schönlein purpura was also characterized [34]. Because IL-1 activity depends on the IL-1 to IL-1ra ratio [35], and as discussed previously, patients carrying IL1RN*2 may have increased production of IL-1ra and IL-1β, it is reasonable to speculate that the administration of IL-1 blockade or IL-1ra enhancer may have a beneficial effect in the treatment of IgAN. In this regard, treatment with IL-1ra in an experimental model of crescentic glomerulonephritis showed a significant reduction of proteinuria and a recovery of normal renal function [11, 36]. In a spontaneously occurring model of IgAN in mice, treatment with IL-1ra was associated with a significant reduction of proteinuria and improvement of renal ^{51}Cr-EDTA clearance [12].

Tumor necrosis factor-α is also a potent cytokine involved in many glomerular diseases, including IgAN [4, 21, 37, 38]. TNF-α may be produced locally in kidney cells [6, 32] or derived from circulating macrophages/monocytes [6, 39] in IgAN. A study of cytokine expression in IgAN patients showed that over 90% were positive for TNF-α, from both peripheral blood mononuclear cells and kidney tissue [6]. More recently, Inaba et al documented a significantly higher serum TNF-α level in IgAN patients with gross hematuria than in those with only microscopic hematuria [40]. TNF-α tended to decrease after the macroscopic hematuria disappeared. It is interesting to find that in our study, patients carrying TNF2 who were supposed to have increased TNF-α transcription [23] were also associated with gross hematuria, thus providing a genetic basis for the previous clinical observation [40].

To our knowledge, the significance of carriage of TNF2

in IgAN patients has not been previously addressed. The carriage of TNF2 has been found to be associated with several inflammatory diseases, including alopecia areata [24], rheumatoid arthritis [25], and systemic lupus erythematosus [26], implicating a pathogenetic role of TNF-α in these diseases. It seemed that the carriage of the TNF2 allele was associated with an increase in disease susceptibility rather than disease activity [24–26]. A study of TNF-α released by cultured monocytes, either spontaneously or induced by lipopolysaccharide in IgAN patients, showed that the level did not appear to correlate with clinical disease activity as indicated by the degree of proteinuria, degree of hematuria, erythrocyte sedimentation rate, and creatinine clearance [39]. Although serum TNF-α was associated with increased mesangial cell proliferation, it was unrelated to the grade of mesangial matrix expansion and the degree of proteinuria [40]. In experimental models of glomerulonephritis, however, the infusion of TNF-α and interferon-γ was shown to cause glomerular endothelial damage and to exacerbate proteinuria [41]. In our study, the carriage of TNF2 was not correlated with disease progression and long-term outcome and did not even correlate with initial disease activity except for gross hematuria, suggesting that TNF-α probably did not play an important role in disease progression in human IgAN. It is likely that an increased TNF-α level may occur only when the precipitating factor of gross hematuria appears, such as an upper respiratory tract infection, and affect kidney function only temporarily. Although gross hematuria has been shown to exacerbate renal function temporarily [42], most studies indicated that the presence of gross hematuria in IgAN was not correlated with a worse prognosis [43–45].

In summary, we have documented an impact of IL-1ra and TNF-α gene polymorphism on the disease susceptibility and clinical picture of human IgAN and have thus highlighted the importance of a genetic basis in the disease pathogenesis and progression. The finding that the carriage of IL1RN*2 may predispose people to a poor long-term outcome supports the relevance of IL-1ra therapy in the treatment of IgAN.

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